

In the Claims

The following list of pending claims replace any prior list of claims.

Listing of Claims

1. (Withdrawn) A method for the preparation of evolved microorganisms permitting a modification of metabolic pathways, characterized in that it comprises the following steps:
 - a) preparing a modified microorganism by genetic modification of cells of an initial microorganism so as to inhibit the production or consumption of a metabolite when that microorganism is grown on a defined medium, thereby impairing the ability of that microorganism to grow;
 - b) culturing the modified microorganism thereby obtained on said defined medium to cause it to evolve, where the defined medium can contain a co-substrate to allow such evolution;
 - c) selecting a modified microorganism able to grow on said defined medium, if necessary with a co-substrate.
2. (Withdrawn) The method as claimed in Claim 1, characterized in that the metabolic pathway is chosen from among: biosynthesis pathways of amino acids, synthesis pathways of nucleic acids, synthesis pathways of lipids, and metabolism pathways of sugars.
3. (Withdrawn) The method as claimed in Claim 2, characterized in that the modified metabolic pathway is a biosynthesis pathway of amino acids.
4. (Withdrawn) The method as claimed in Claim 3, characterized in that the modified metabolic pathway is a biosynthesis pathway of an amino acid chosen from among: methionine, cysteine, threonine, lysine, and isoleucine.
5. (Withdrawn) The method as claimed in Claim 2, characterized in that the modified metabolic pathway consumes NADPH.

6. (Withdrawn) The method as claimed in Claim 1, characterized in that the modification made in step a) favors the reduction of NADP to NADPH, possibly by limiting the oxidation of NADPH to NADP.

7. (Withdrawn) The method as claimed in Claim 1, characterized in that the evolved microorganism possesses at least one evolved gene coding for an evolved protein, the evolution of which replaces the inhibited metabolic pathway by a new metabolic pathway.

8. (Withdrawn) The method as claimed in Claim 1, characterized in that it includes an additional step a1), of introducing at least one heterologous gene coding for a heterologous protein, which heterologous gene is to allow the evolution of a new metabolic pathway, preparatory to step b) in which the modified microorganisms are cultured.

9. (Withdrawn) The method as claimed in Claim 7, characterized in that it includes a step d) of isolating the evolved gene coding for the evolved protein.

10. (Withdrawn) The method as claimed in Claim 9, characterized in that the evolved gene is introduced, in an appropriate form, into a production microorganism intended for the production of the evolved protein.

11. (Withdrawn) An evolved microorganism obtainable by a method according to Claim 1.

12. (Withdrawn) An evolved microorganism according to Claim 11, characterized in that the microorganism is the strain *E. coli* K183 with a modified "methionine synthase" activity, registered April 2, 2003 under the number I-3005 at the CNCM.

13. (Original) A method for the preparation of an evolved protein, wherein the evolved microorganism according to Claim 11 is cultivated in a culture medium appropriate for the production of the evolved protein.

14. (Original) The method as claimed in Claim 13, characterized in that the produced evolved protein is purified.

15. (Withdrawn) An evolved gene coding for an evolved protein obtainable by a method according to Claim 9.

16. (Withdrawn) An evolved protein obtainable by a method according to Claim 13.

17. (Withdrawn) An evolved protein according to Claim 16, characterized in that the enzyme has a modified "methionine synthase" activity and is chosen from cystathionine- γ -synthases and acylhomoserine sulphydrylases with modified "methionine synthase" activity.

18. (Withdrawn) An evolved protein according to Claim 17, characterized in that the cystathionine- γ -synthase with non-modified "methionine synthase" activity before the evolution is selected from cystathionine- γ -synthases corresponding to PFAM with the reference PF01053 and COG with the reference CPG0626.

19. (Withdrawn) An evolved protein according to Claim 18, where the cystathionine- γ -synthase with non-modified "methionine synthase" activity before the evolution, comprises the following amino acid sequence in its C-terminus (conserved region 1)

X1-X2-X3-L-G-X4-X5-X6-X7-X8-X9

in which:

X1 represents A,G,S, preferentially A;

X2 represents E,V,P,T, preferentially E;

X3 represents S,T,N, preferentially S;

X4 represents G,D,A,H,T, preferentially G;

X5 represents V,A,T,H,N, preferentially V;

X6 represents E,R,K,F, preferentially E;

X7 represents S,T, preferentially S;

X8 represents L,I,V,A, preferentially L; and

X9 represents I,V,A,T, preferentially I

corresponding to residues 324 to 334 of the cystathionine- γ -synthase sequence of *E. coli* K12, represented by SEQ ID NO 6.

20. (Withdrawn) An evolved protein according to Claim 18, characterized in that the cystathionine- γ -synthase with non-modified "methionine synthase" activity before the evolution, comprises the following amino acid sequence in its N-terminus (conserved region 2):

X10-X11-Y-X12-R-X13-X14-X15-X16-X17-X18

in which:

X10 represents A, H, Y, F, L, K, preferentially A;

X11 represents Y, E, D, K, R, V, I, preferentially Y;

X12 represents S,A,T,P,G, preferentially S;

X13 represents I,S,T,R,E,F,W,D, preferentially S;

X14 represents S,G,A,I,E,N,K,P, preferentially G;

X15 represents N,H,Q,S, preferentially N;

X16 represents P,D,L, preferentially P;

X17 represents T,M,N,G,S, preferentially T; and

X18 represents R,L,V,S,W,E, preferentially R

corresponding to residues 44 to 54 of the cystathionine- γ -synthase sequence of *E. coli* K12, represented by SEQ ID NO 6.

21. (Withdrawn) An evolved protein according to Claim 18, characterized in that it comprises at least one mutation in its C-terminal part and/or at least one mutation in its N-terminal part.

22. (Withdrawn) An evolved protein according to Claim 21, characterized in that the mutation consists in replacing an acidic amino acid, which interacts with the co-substrate cysteine in the non-modified enzyme, by a non-polar amino acid, selected from the residues glycine, alanine, leucine, isoleucine, valine, phenylalanine and methionine.

23. (Withdrawn) An evolved protein according to Claim 22, characterized in that the mutation in the C-terminal part of the cystathionine- γ -synthase is introduced among the acidic amino acids of "conserved region 1", particularly into residue X2.

24. (Withdrawn) An evolved protein according to Claim 23, characterized in that it comprises the following amino acid sequence in its C-terminal part:

X1-X2-X3-L-G-X4-X5-X6-X7-X8-X9

in which:

X1, X3, X4, X5, X6, X7, X8 et X9 are defined above and

X2 represents G,A,L,I,V,F,M, preferentially A

corresponding to residues 324 to 334 of the cystathionine- γ -synthase sequence of *E. coli* K12, represented by SEQ ID NO 8.

25. (Withdrawn) An evolved protein according to Claim 24 characterized in that it comprises the following amino acid sequence in its C-terminal part:

A-A-S-L-G-G-V-E-S

corresponding to residues 324 to 332 of the cystathionine- γ -synthase sequence of *E. coli* K12, represented by SEQ ID NO 8.

26. (Withdrawn) An evolved protein according to Claim 25 characterized in that the cystathionine- γ -synthase with modified « methionine synthase » activity comprises the amino acid sequence represented by SEQ ID NO 8.

27. (Withdrawn) An evolved protein according to Claim 18, characterized in that the mutation in the N-terminal part of the cystathionine- γ -synthase is introduced among the acidic amino acids of the conserved region 2, as defined above, in particular into residues X11 and/or R and/or X13.

28. (Withdrawn) An evolved protein according to Claim 27 characterized in that the cystathionine- γ -synthase with modified « methionine synthase » activity comprises the following amino acid sequence in its N-terminal part:

X10-X11-Y-X12-X19-X13-X14-X15-X16-X17-X18

in which:

X7, X9, X12, X14, X15, X16, X17 et X18 are defined above,

X11 is defined in Claim 15 or represents a non-polar amino acid,

X13 is defined in Claim 15 or represents a non-polar amino acid,

X19 is R or represents a non-polar amino acid, and

at least one of X11, X13 et X19 represents a non-polar amino acid,

where the non-polar amino acids are chosen independently among the residues glycine, alanine, leucine, isoleucine, valine, phenylalanine or methionine.

29. (Withdrawn) An evolved protein according to Claim 16, characterized in that the initial enzyme without mutations, before the evolution, catalyzes a sulphydrylation reaction in the presence of H₂S.

30. (Withdrawn) An evolved protein according to Claim 29, characterized in that the initial enzyme without mutations, before the evolution has O-acyl-L-homoserine sulphydrylase activity.

31. (Withdrawn) An evolved protein according to Claim 30, characterized in that the initial enzyme with O-acyl-L-homoserine sulphydrylase activity is chosen among the O-acyl-L-homoserine sulphydrylases corresponding to PFAM reference PF01053 and COG reference COG2873.

32. (Withdrawn) An evolved protein according to Claim 31, characterized in that the initial enzyme is chosen among the following acylhomoserine sulphydrylases:

NP_785969 O-acetylhomoserine (thiol)-lyase, *Lactobacillus plantarum* WCFS1;

AAN68137 O-acetylhomoserine sulphydrylase, *Pseudomonas putida* KT2440;
NP_599886 O-acetylhomoserine sulphydrylase, *Corynebacterium glutamicum* ATCC13032;
NP_712243 acetylhomoserine sulphydrylase, *Leptospira interrogans* serovar lai str. 56601;
BAC46370 O-succinylhomoserine sulphydrylase, *Bradyrhizobium japonicum* USDA110;
AAO57279 O-succinylhomoserine sulphydrylase, *Pseudomonas syringae* pv. tomato str.
DC3000;
NP_284520 O-succinylhomoserine sulphydrylase [*Neisseria meningitidis* Z2491; and
AAA83435 O-succinylhomoserine sulphydrylase (*P. aeruginosa*).

33. (Withdrawn) An evolved protein according to Claim 31, characterized in that the initial enzyme before the evolution, is O-acyl-L-homoserine sulphydrylase encoded by the *metY* gene of *Corynebacterium*.

34. (Withdrawn) An evolved protein according to Claim 33, characterized in that the O-acyl-L-homoserine sulphydrylase is encoded by the *metY* gene of *Corynebacterium glutamicum* (Genbank AF220150).

35. (Withdrawn) A method of biotransformation comprising culturing an evolved microorganism according to Claim 11 under conditions for biotransformation by fermentation or bioconversion.

36. (Withdrawn) A biotransformation method according to Claim 35 where the biotransformation is depending on NADPH dependant enzymes.

37. (Withdrawn) A method according to Claim 36 in which the NADPH-dependant enzymes have evolved substrate specificity.